Familial and Congenital Polycythemia in Three Unrelated Families


Three families with polycythemia inherited through apparently different modes are described. Secondary causes of polycythemia were ruled out. Erythropoietin (EPO) levels were normal or low, even after phlebotomy. In vitro erythroid colony growth in standard assay cultures containing EPO was normal; however, in the absence of added EPO, a few progenitors from most of the affected individuals were able to generate recognizable colonies of mature erythroblasts, although these were smaller and proportionately less numerous than seen in polycythemia vera (PV). To search for EPO-receptor changes as a possible pathophysiologic mechanism, we examined, by Southern blot analysis, genomic DNA samples from affected and nonaffected family members, as well as three patients with PV. Two different probes, derived from the human EPO-receptor, were used. We found no evidence for chromosomal rearrangements or gene amplification in hereditary polycythemia or PV patients. Further, no nucleotide sequences were found that were homologous to the Friend spleen focus-forming virus glycoprotein gp55, which has been shown to bind to and activate the murine EPO-receptor. Functional studies examining number and binding affinity of the EPO-receptor on erythroid progenitors from three hereditary polycythemia patients demonstrated no abnormalities. We conclude that the mechanism(s) for the erythrocytosis in familial and congenital polycythemia and in PV may not involve the EPO-receptor and, therefore, may result from alterations of postreceptor responses.

POLYCYTHEMIA or erythrocytosis, the term used to describe any condition where there is a significant expansion of the red blood cell mass, occurs in a number of disorders.1,2 The most common forms of absolute polycythemia are acquired and occur relatively late in life. They include those caused by hypoxia from underlying cardiopulmonary disease, from living at high altitudes, and from defective oxygen transport; those associated with erythropoietin (EPO)-producing tumors; and those resulting from the selective overgrowth of a neoplastic hematopoietic stem cell whose erythroid progeny are able to differentiate in the absence of EPO.3-5 In contrast, familial forms of polycythemia may be present in childhood as well as adulthood and are more rare.10 Nichamin first described familial polycythemia in 1908.11 Over the next 60 years many cases were reported.10,12 Most cases of familial and congenital polycythemia were associated with hemoglobin (Hb) mutations that result in an increased affinity for oxygen.10,13 More rarely, abnormal levels of 2,3-DPG were associated with familial polycythemia.14-17 Some case reports of polycythemia, which do not appear to be associated with a Hb mutation, have not been further characterized.18 However, since the late 1960s several cases of familial and congenital polycythemia not associated with Hb mutations or enzyme abnormalities have been distinguished on the basis of EPO production. Several cases, including both autosomal dominant and recessively inherited types of polycythemia, have been associated with an increased stimulation of an otherwise normal hematopoietic system, on the basis of the demonstration of elevated levels of serum EPO.19-23 There have also been a few reports of polycythemic patients who appeared to have an increased EPO production without evidence of a secondary cause of their erythrocytosis nor any family history of polycythemia. It is unclear as to whether the disorders of these latter individuals were congenital because neonatal Hb determinations were not performed.24-28

All of the above examples of polycythemias appear to be “secondary” in that the erythropoiesis is driven by an extrinsic stimulus on the erythroid progenitors, such as EPO. However, there have also been a few examples of familial polycythemia without evidence of excessive EPO production.29-34 The disorder in one family was described as a “polycythemia vera (PV) variant.”32 The distinction from PV is an important one since familial polycythemia vera has been described.35 The polycythemic subjects in some of the other families reported previously did not fit the criteria established for PV,3 or other causes of familial polycythemia, and were inherited in an autosomal dominant fashion.30,32-34 Because of a lack of evidence of secondary polycythemia and no evidence of increased EPO bioactivity, these latter families may represent a true or “primary” polycythemia.

In this report, we present detailed clinical and laboratory findings, including EPO-receptor studies, on another three unrelated white families with familial and congenital forms of “primary” polycythemia. In two of these families, the inheritance of the condition appeared to fit an autosomal dominant pattern. In the other family, the patient was...
found to have congenital polycythemia although his parents were both normal.

MATERIALS AND METHODS

Case Reports

Family 1. The propositus (II,1), now 9 years old, has been observed at the University of Alabama at Birmingham since he was only a few weeks old (Fig 1A). Apart from a slightly ruddy complexion, the patient has been otherwise clinically asymptomatic. He has had no splenomegaly by examination. He has felt well the entire time that he has been studied and has not required any treatment for his erythrocytosis.

His parents and brother were found to be normal; however, the mother did deliver a still-born male child 3 years after the birth of the propositus.

Family 2. The propositus (V,1), now 18 years old, has been observed at the University of Alabama at Birmingham for the last 8 years (Fig 1B). Her Hb concentration has ranged from 16.9 to 20.3 g/dL. The spleen has not been enlarged. She has minimal symptoms consisting of intermittent headaches, numbness of hands and feet, and difficulty concentrating. She has a somewhat ruddy complexion. Some improvement in her symptoms was noted after phlebotomy several years ago, but not during the phlebotomies performed over the last 3 years.

The father of the propositus (IV,2), who has also been observed at the University of Alabama at Birmingham for the last 6 years, was initially diagnosed in 1975. His symptoms have included mild headaches, dizzy spells, weakness, mild indigestion, and occasional nocturnal leg cramps. The patient has been described as plethoric. His spleen has not been enlarged, and he has noted inconsistent improvement with phlebotomy.

The paternal aunt (IV,5) of the propositus has also been affected. She was first diagnosed in 1980 when she was pregnant with her third child. Her symptoms include tightness in the neck, headaches, and lethargy. The patient has not had a ruddy complexion. Her spleen has not been enlarged. She has not felt that phlebotomy helped her symptoms. This patient has three children, two of whom are also affected. The eldest son (V,4) is now 17 years old. He also complained of intermittent headaches, numbness of hands and feet, and difficulty concentrating. He has a slight ruddy complexion. His spleen has not been enlarged. He has felt his symptoms were exacerbated by phlebotomy. The second son (V,5), now age 14, has symptoms of chronic headaches and bleeding hemorrhoids. An elevated Hb concentration was found 10 days after birth and has persisted ever since. His spleen has not been enlarged. The youngest child (V,6), a daughter now 9 years old, does not have polycythemia.

All of the affected family members have been observed for at least 6 years. Some were investigated for elevated Hb concentrations over the last 30 years at various hematology centers in Louisiana and Mississippi. None have had any vaso-occlusive or bleeding incidents. None have required any other therapy except phlebotomy. Some were mistakenly initially diagnosed as polycythemia vera, and one declined offered therapy with radioactive 32P. Eleven family members besides those described above have also had Hb measurements and sometimes more extensive studies performed. Eight of these additional 11 members have shown an elevated Hb concentration, the other three had Hb concentrations within the normal range for their gender and age.

Family 3. The propositus and his father both were found to have an elevated Hb concentration (see Fig 1C). The propositus first presented in 1987 after a minor episode of epistaxis. Both patients have a ruddy complexion and symptoms of headaches and energy and irritability, and has been described as having a somewhat ruddy complexion. Some improvement in her symptoms was noted after phlebotomy several years ago, but not during the phlebotomies performed over the last 3 years.

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lethargy. Splenomegaly could not be assessed clinically in either patient because of their obesity. Both the propositus and his father have felt some improvement in symptoms with phlebotomy. Screening of family members showed nine other subjects with a history of polycythemia, with a suggestive history in an additional four.

Detailed interrogation of the families has not shown any evidence supporting consanguinity, nor has there been any evidence of a genetic relationship between any of these three families or the two families that we have described previously.

Laboratory Studies

P50 values were calculated from venous blood gases. Red blood cell (RBC) and plasma volumes were estimated by separately labeling RBCs and albumin by standard methods. Measurements of RBC 2,3-DPG were performed as described by Beutler. Serum EPO levels were determined by radioimmunossay (RIA), or by Clingen EIA (AMGEN, Thousand Oaks, CA), and in some cases, also by biologic assay using ex-hypoxic polycythemic mice.

Bone marrow and peripheral blood samples were taken after obtaining informed written consent from seven study subjects, two nonaffected family members, and several PV and nonpolycythemic controls. Bone marrow cells were aspirated from the posterior iliac spine into sterile plastic tubes containing 5 mL of RPMI 1640 (Sigma, St Louis, MO) with 5% fetal calf serum (FCS) and 200 U of preservative-free heparin. Peripheral blood samples were also collected into heparin. Both marrow and blood samples were then coded and shipped at room temperature by overnight courier to Vancouver or Seattle for further studies.

Erythroid colony assays were performed to assess endogenous colony formation and sensitivity to EPO. These assays were performed in Vancouver by isolating nucleated marrow cells and light density (<1.077 g/cm3) peripheral blood cells and plating them in standard methylcellulose cultures containing 30% FCS, 1% bovine serum albumin (BSA), 10% agar-stimulated human leukocyte-conditioned medium, 10-5 M/L 2-mercaptoethanol, and either 3 to 5 U/mL or no EPO, unless indicated otherwise. In cultures of earlier samples, partially purified human urinary EPO was used. In cultures of later samples, greater than 90% pure human urinary EPO was used. In our experience at the time these studies were performed, agar-stimulated human leukocyte-conditioned medium served as the best source of non-EPO growth factors (by comparison with recombinant interleukin-3 [IL-3], granulocyte-macrophage colony-stimulating factor [GM-CSF], or G-CSF) to obtain maximum plating efficiency of all classes of erythroid progenitors. Cultures were then incubated at 37°C in a humidified atmosphere of 5% CO2 in air before assessment of colony formation in situ. Smaller erythroid colonies, derived from colony-forming unit-erythroid (CFU-E) and “mature” burst forming unit (BFU-E) were scored after 10 to 12 days in culture, using criteria from previous results. Larger erythroid colonies (from more primitive BFU-E), all GM colonies (from CFU-GM), and colonies containing both erythroid and granulopoietic elements (from CFU-GEMM) were scored in the same cultures after another 8 to 10 days using established criteria for colony classification. The propositus (and the parents) from Family 1 was also tested on two different occasions, at the age of 2 years, in a different laboratory than that above to test for erythroid progenitor characteristics, using a modification of the above assays as we have described previously.

EPO-receptor binding studies were performed to assess EPO binding characteristics both to populations enriched in late erythroid progenitors (CFU-E) and to erythroblasts. Autoradiographic analysis was used to compare 125I-EPO binding to enriched CFU-E populations from affected and unaffected family members. Peripheral blood mononuclear cells were cultured at a concentration of 5 x 10^9/mL in 1.3% methylcellullose in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 30% heat inactivated FCS, 1% deionized BSA, 1.5 U/mL partially purified human EPO, and 2 U/mL recombinant human IL-3 (Genetics Institute, Cambridge, MA). After 7 days of culture, enriched CFU-E populations were harvested by plucking small young erythroid bursts containing less than 100 cells. The cells were washed twice and incubated in IMDM plus 5% FCS for 90 minutes at 37°C to permit internalization and degradation of EPO bound to cell surface receptors, then incubated with 1 nmol/L high-specific activity 125I-EPO (4,000 Ci/mmol; Amersham, Arlington Heights, IL) for 1 hour at 37°C in the presence of 0.2% sodium azide and processed for autoradiography as described. The slides were exposed for 2 to 4 weeks. Specific binding was determined by counting the grains overlying the cells and in an area immediately adjacent to each cell, and subtracting the grain count in the adjacent area. To determine the fraction of CFU-E, a portion of the pooled day 7 colonies were replated in methylcellulose as described above. CFU-E colonies (well-hemoglobinized red colonies containing 30 to 100 cells) were counted 7 days later. As an alternative approach to that described above for obtaining CFU-E enriched populations, direct immune adherence of peripheral blood mononuclear cells from affected and unaffected family members was performed with the monoclonal antibody (MoAb) SR-1. Cells adhering to SR-1 coated plates are enriched in BFU-E. The SR-1 MoAb recognizes the c-kit receptor on BFU-E, CFU-GM, and BFU-E. The SR-1 adherent cells were cultured for 6 days in suspension, in IMDM supplemented with 25% FCS, 1% BSA, 50 U/mL IL-3, 50 ng/mL stem cell factor (AMGEN), and 2 U/mL EPO, then were washed, incubated with high-specific activity 125I-EPO, and processed for autoradiography as described above. Under these conditions, the populations of cells after 6 days in culture are highly enriched for BFU-E, and young erythroblasts in addition to BFU-E. The fraction of CFU-E was determined by replating the cells in methylcellulose as described above.

Scatchard analysis of 125I-EPO binding to erythroblasts was performed to determine the number of receptors per cell and the dissociation constant (kd). To obtain populations of pure erythroblasts, after 10 to 14 days incubation in methylcellulose cultures hemoglobinized erythroid colonies were plated and incubated in suspension in IMDM supplemented with EPO and IL-3 (as above) for 24 hours at 37°C, then washed twice (as above) and incubated in IMDM plus 5% FCS for 90 minutes at 37°C. This approach can provide up to 40 x 10^9 erythroblasts. EPO receptors were quantitated by incubating the erythroblasts with varying amounts of 125I-EPO (300 to 900 Ci/mmol; Amersham) for 6 hours at 15°C to achieve equilibrium binding. The cells (1 to 2 x 10^9) were added to a premixed solution of 125I-EPO (32 pmol/L to 2.2 nmol/L) with or without a 100-fold molar excess of unlabeled purified human recombinant EPO in buffer consisting of RPMI supplemented with 1% BSA and 50 mmol/L HEPES (pH 7.4). All samples were run in duplicate. At the conclusion of the binding experiment, cell-associated 125I-EPO was separated from free 125I-EPO by sedimenting the cells through phthalate oil using a Beckman Microfuge 11 (Fullerton, CA) operated in a 4°C cold room. The cell pellets were clipped off, and radioactivity in both the pellets and supernatants were measured in a Packard gamma counter (Downers Grove, IL) with an efficiency of 66%. The equations for one or two classes of receptors were fitted to the data using the ligand program.

Paternity studies were performed by examination of the highly variable genomic locus hMFl and Southern blotting. These studies included unique, nonpaternal fragments as well as unique paternal-specific and maternal-specific fragments. These were compared by their established incidence in a white population.
Extraction of DNA from peripheral blood cells and Southern blot analyses were performed using standard techniques. We used two genomic EPO-receptor probes, the first containing about 1 kb of the human EPO-receptor gene that contained most of the coding region. Later, because it was found that truncation of the carboxy terminus of the EPO-receptor resulted in increased EPO sensitivity, we used a second 0.5-kb genomic probe containing the 3' untranslated region and the carboxy terminus of the human EPO-receptor. To quantify the gene dose, the nitrocellulose filters were rehybridized with an unrelated genomic probe for the nerve growth factor receptor designated E51.5. The medium stringency conditions were hybridization at 37°C with 40% formamide and 1.5 mol/L NaCl in the hybridization solution; hybridization at 42°C and washing at 65°C with 2X SSC (0.3 mol/L sodium chloride, 0.3 mol/L sodium citrate). The high stringency conditions included hybridization at 37°C with 35% formamide and 1.75 mol/L NaCl, followed by washing at room temperature in 5X SSC and overnight autoradiography. The high background was then reduced by repeating washing of the membrane first at 37°C and overnight autoradiography. The human genomic DNA samples were examined with the enzymes BamHI, BglI, EcoRI, PstI, SpHl, SstI, TaqI, and XhoI, according to standard techniques. In each Southern blot analysis, genomic DNAs from one affected and one nonaffected individual from each family and three individuals with documented PV were compared with controls.

Table 1. Clinical and Laboratory Data From Representative Polycythemic Patients and Normal Family Members

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Sex</th>
<th>Hb (g/dL)</th>
<th>Hct (%)</th>
<th>WBC (10⁹/L)</th>
<th>Platelets (10⁹/L)</th>
<th>P50</th>
<th>2,3-DPG*</th>
<th>RBC Volume</th>
<th>Plasma Volume</th>
<th>Bone Marrow</th>
<th>Epo Level (mU/mL)</th>
<th>2,3-DPG* (nmol/gHb)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Family 1:</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>AFFECTED INDIVIDUAL</td>
<td>9</td>
<td>M</td>
<td>20.5</td>
<td>59.3</td>
<td>6.2</td>
<td>315</td>
<td>30.5</td>
<td>94.5</td>
<td>37.7†</td>
<td>47.9#</td>
<td>IRON DEFICIENT</td>
<td>165</td>
<td>13,051</td>
</tr>
<tr>
<td>Normal family member</td>
<td>34</td>
<td>F</td>
<td>13.7</td>
<td>40.3</td>
<td>6.0</td>
<td>293</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Normal</td>
<td></td>
<td>22†</td>
<td>13,932</td>
</tr>
<tr>
<td><strong>Family 2:</strong></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>AFFECTED INDIVIDUAL</td>
<td>18</td>
<td>F</td>
<td>20.3</td>
<td>62.7</td>
<td>6.7</td>
<td>218</td>
<td>27.4</td>
<td>97</td>
<td>30.8†</td>
<td>34.1‡</td>
<td>RELATIVE ERYTHROID HYPERPLASIA</td>
<td>6</td>
<td>13,686</td>
</tr>
<tr>
<td>Normal family member</td>
<td>9</td>
<td>F</td>
<td>13.3</td>
<td>37.2</td>
<td>6.9</td>
<td>383</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Family 3:</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>AFFECTED INDIVIDUAL</td>
<td>13</td>
<td>M</td>
<td>18.4</td>
<td>66.7</td>
<td>5.3</td>
<td>260</td>
<td>28.4</td>
<td>99.1</td>
<td>2,136**</td>
<td>2188††</td>
<td>Normal</td>
<td>7</td>
<td>12,461</td>
</tr>
</tbody>
</table>

RBC and plasma volumes were calculated in different labs and thus there are different normal ranges.

*Normal range 8.530-16.030 nmol/g Hgb.
†Normal range 20-30 mL/kg.
‡Normal mean 52.1 ± 4.3 mL/kg.
§Confirmed normal level by RIA and bioassay (two independent labs).
||ND, not done.
¶Normal range 10-26 mL/mL.
#Normal range 30-46 mL/kg.
**Calculated upper limit of normal for size and gender: 1,718 mL.
††Calculated upper limit of normal for size and gender: 2,477 mL.

RESULTS

Clinical and Laboratory Findings

Clinical and laboratory findings on the members of the polycythemic families studied here are summarized in Table 1. For simplicity, we have included only one representative polycythemic patient from each family, along with a respective normal family member in this table. Family members studied in detail were: family 1 (one affected, three normal), family 2 (five affected, two normal), and family 3 (two affected). In addition to the findings presented in Table 1, erythrocyte adenosine triphosphate (ATP) levels, leukocyte alkaline phosphatase scores, vitamin B12 levels, and bone marrow chromosome analysis in some individuals (at least one affected member of each family) were also performed. The results of all of these additional studies were normal (data not shown). In addition to the individuals studied in detail, 11 other members of family 2 and 9 other members of family 3 had at least Hb measurements performed. The ranges for some of the distinguishing laboratory measurements in affected members in families 2 and 3 are as follows: Hb = 16.9 to 20.3 g/dL (family 2), 18.4 to 22.1 (family 3); P50 = 27.14 to 27.77 (family 2), 28.2 to 28.4 (family 3); EPO level = 5 to 8 mU/mL (family 2), 5 to 7 (family 3); and 2,3-DPG = 12,648 to 14,218 nmol/gHb (family 2), 12,461 to 13,131 (family 3). White blood cell (WBC) and platelet measurements were normal in all members of family 2 and family 3, both affected and normal. Other causes of secondary polycythemia were ruled out in all affected individuals by demonstration of normal arterial blood oxygen saturation. P50 values calculated from venous blood gases were normal in affected individuals (as above) as well as all normal family members tested. Because P50 values were obtained from a single
point and hence full oxygen dissociation curves were not discernable, erythrocyte 2,3-DPG and ATP levels were also obtained to exclude polycythemia-associated enzyme abnormalities such as diphosphoglycerate mutase deficiency.\(^{14,17}\) All individuals demonstrated 2,3-DPG levels in the normal range (as above and Table 1). The RBC volume was elevated in all affected individuals that were tested, when corrected for their age and gender\(^{63-65}\); while their plasma volumes were normal.

**Serum EPO Levels**

The affected individual in family 1 had a normal serum EPO level (16 mU/mL by RIA) (Table 1) when initially tested and a level below the normal mean when tested later (Fig 2). For the five affected members tested in family 2, EPO levels (5 to 8 mU/mL) were below the normal range as was also found for the two affected members in family 3 who have been studied extensively (EPO levels 5 and 7 mU/mL). To exclude the possibility that a mutated EPO protein might have markedly elevated bioactivity, yet have a normal level as measured by RIA, serum EPO levels in families 1 and 2 were also tested by bioassay in ex-hypoxic polycythemic mice\(^4\) and found in every case to be below the limit of detectable activity. However, it must be pointed out that the lower limit of detectability of this assay is 50 mU/mL, thus it is relatively insensitive. Nevertheless, marked elevations in EPO bioactivity were ruled out.

Serum EPO levels were also measured during a period of intensive phlebotomy to reduce the Hb by approximately 20% from the propositus individuals from families 1 and 2. Points of analysis included serum samples from just before phlebotomy, 1 to 2 hours post-phlebotomy, 6 to 7 days post-phlebotomy, and more distant samples (Fig 2). EPO levels in these cases were determined using the Clinigen EIA (AMGEN). In studies of 262 normal individuals, the mean serum EPO level using the Clinigen EIA was 6.5 mU/mL. The correlation coefficient for the Clinigen EIA and the AMGEN RIA is 0.99.\(^{66}\)

**Progenitor Studies**

All affected family members studied showed a normal or low concentration of all types of clonogenic progenitors in both blood and bone marrow samples tested, but a similar decrease in progenitor levels was also found in concurrently shipped controls (Table 2). Most affected patients (eg, the propositus from family 1, two subjects from family 2, and one from a previously studied family restudied at this time\(^3\) [Table 2]) showed low levels of EPO-independent growth of CFU-E on some occasions, although at other times this was not detected. Complete EPO dose-response curves were generated for the affected individual studied from family 1 at several different ages, the first occurring at age 2. These studies were performed in two different labs with methods as stated above and as described previously.\(^3\) In each instance the dose response was shifted slightly leftward (greater responsiveness to lower doses of EPO) as compared with an average normal curve\(^6\) so that some hemoglobinized colonies of CFU-E origin were still detectable under conditions where no exogenous EPO was added (data not shown). There were variations observed in the degree of leftward shift of the dose response. Previous studies of these conditions have suggested that the FCS used may introduce an EPO concentration of up to 0.001 U/mL.\(^67\) Accordingly, some CFU-E in a population with a
Table 2. Erythroid Progenitor Analysis From Representative Polycythemic Patients and Normal Family Members (same individuals as in Table 1)

<table>
<thead>
<tr>
<th>Family 1:</th>
<th>Marrow (per 2 \times 10^6 cells)</th>
<th>Blood (per 4 \times 10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Date + -</td>
<td>+ -</td>
</tr>
<tr>
<td><strong>Affected individual</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4/84</td>
<td>410 8 129 0</td>
<td>26 0 102 0</td>
</tr>
<tr>
<td>5/84</td>
<td>ND ND ND ND</td>
<td>9 0 92 0</td>
</tr>
<tr>
<td>8/87</td>
<td>ND ND ND ND</td>
<td>5 0 34 0</td>
</tr>
<tr>
<td><strong>Normal family member</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4/84</td>
<td>254 0 102 0</td>
<td>19 0 129 0</td>
</tr>
<tr>
<td>4/87</td>
<td>ND ND ND ND</td>
<td>5 0 73 0</td>
</tr>
<tr>
<td><strong>Family 2:</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>Affected individual</strong></td>
<td></td>
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<tr>
<td>4/84</td>
<td>356 0 82 0</td>
<td>3 0 37 0</td>
</tr>
<tr>
<td>5/84</td>
<td>ND ND ND ND</td>
<td>2 0 4 0</td>
</tr>
<tr>
<td><strong>Normal family member</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8/87</td>
<td>112 0.3 80 0.5</td>
<td>No growth</td>
</tr>
<tr>
<td><strong>Family 3:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Affected individual</strong></td>
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<td></td>
</tr>
<tr>
<td>6/87</td>
<td>321 0 286 0</td>
<td>2 0 32 0</td>
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<tr>
<td><strong>Previously studied autosomal dominant polycythemia</strong></td>
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<td></td>
</tr>
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<td>4/87</td>
<td>ND ND ND ND</td>
<td>11 1 83 2</td>
</tr>
<tr>
<td>8/87</td>
<td>ND ND ND ND</td>
<td>14 2 32 4</td>
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<tr>
<td><strong>Control samples, shipped concurrently PV patients</strong></td>
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<tr>
<td>No. 2</td>
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<td><strong>Representative nonpolycythemic controls</strong></td>
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<td>8/87 131 0 76 0</td>
<td>ND ND ND ND</td>
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Abbreviations: ND, not done; PV, polycythemia vera; +, grown in the presence of EPO; -, grown in the absence of EPO.

*Data from Prchal et al.*

sufficiently increased EPO sensitivity (or decreased EPO requirement) would be expected to form recognizable colonies under these conditions. In contrast, there was no erythroid colony formation in cultures without EPO that were set up with concurrently shipped samples from either unaffected family members or nonpolycythemic controls. Conversely, all cultures set up with concurrently shipped PV cells sent as positive controls showed readily detectable EPO-independent CFU-E and BFU-E at proportionately higher levels (Table 2).

**EPO-Receptor Studies**

A representative Southern analysis of DNA samples using a human EPO-receptor probe is illustrated in Fig 3. In testing samples obtained from normal individuals, affected individuals, nonaffected family members, and PV patients, no evidence of gene amplification, chromosomal rearrangements, deletions, or insertions could be found in the EPO-receptor using the enzymes BamHI, BglII, EcoRI, Pst I, Sph I, Sst I, Taq I, and Xba I. Further, in these 10 subjects from seven unrelated families, no examples of restriction fragment length polymorphisms (RFLP) were detected using the enzymes and genomic probes described above. Although a slight variation of signal intensity is seen in Fig 3, reprobing of the membranes with an unrelated probe demonstrated that this apparent variation was an artifact of DNA loading. These studies indicate that the EPO-receptor gene is present as a single copy in the human genome.

No nucleotide sequences homologous to the SFFV envelope glycoprotein gp55 were found when examined under several different stringency conditions. Under low stringency conditions, nonspecific DNA binding was evident as a uniform smear on the autoradiographs. This binding was progressively removed with successive washings and no specific binding was demonstrable.

Cell surface EPO-receptors were quantitated by functional studies to search for more subtle abnormalities of the EPO-receptor on (1) the progeny of peripheral blood BFU-E from the affected individual from family 1, the propositus from family 2, and the propositus of a previously described family; and (2) in CFU-E-enriched populations from the affected individual from family 1, the propositus from family 2, as well as a normal family member from each family. The number and binding affinity of the EPO-receptor on the BFU-E-derived erythroblasts from the three patients were similar to those of normal adults. In each case, a single class of binding sites was found (Fig 4). Analysis of binding using a Klotz plot showed that receptor saturation was achieved, arguing against a second...
lower affinity site. After 15 days of culture the erythroblasts from the affected individual from family 1 expressed 154 receptors/cell with a kd of 279 pmol/L. This individual was restudied 2 years later and his BFU-E-derived erythroblasts demonstrated similar results with 248 receptors/cell with a kd of 243 pmol/L. This individual’s normal father was studied in parallel as a control and his erythroblasts showed 172 receptors/cell after 14 days of culture with a kd of 322 pmol/L. These results are illustrated in Fig 4.

Additionally, the results from both the affected individual and his normal father are similar to other studies of normal individuals (235 ± 50 receptors/cell; kd 230 ± 5 pmol/L; n = 3). Likewise, the propositus from family 2 yielded results similar to normals after 14 days of culture of peripheral blood BFU-E with 378 receptors/cell and a kd 405 pmol/L. The peripheral blood BFU-E-derived erythroblasts from the propositus of a previously described family showed 893 receptors/cell with a kd of 206 pmol/L at day 11 of culture. It is important to note that BFU-E-derived erythroblasts from normal adults, cultured for 9 or 14 days, displayed approximately 1,100 or 300 receptors per cell, respectively, with a binding affinity of 220 pmol/L. Thus, the relatively high receptor expression on the peripheral blood erythroblasts from the propositus of this previously studied family reflects the relative immaturity of day 11 erythroblasts as compared with day 14 erythroblasts. EPO-receptors on CFU-E-containing populations were analyzed by two different methods. In the first approach, CFU-E-enriched cells were plucked from young BFU-E colonies on day 7 of culture. These cells were then incubated with $^{125}$I-EPO and autoradiographic analysis was performed. The results showed that a population of cells containing 10.5% CFU-E from the affected individual in family 1 displayed an average of 1.03 specifically bound grains per cell (151 cells counted). Cells from his normal father (9.3% CFU-E) displayed an average of 1.10 grains per cell (124 cells counted). In the second approach, BFU-E-enriched cells were isolated from the peripheral blood of members of family 2 using immune adherence with the MoAb SR-1, which recognizes the c-kit receptor. These cells were
cultured for an additional 6 days in suspension culture, at which time they generated high numbers of CFU-E. These CFU-E-enriched cells were also subjected to autoradiographic analysis using 125I-EPO (Fig 5). This population of cells from the propositus from family 2 demonstrated an average of 2.30 specifically bound grains per cell (188 cells counted, 7.5% CFU-E), while cells from her normal mother exhibited an average of 2.20 grains per cell (151 cells, counted, fraction of CFU-E not evaluated). Thus, CFU-E-enriched cells from affected and normal family members bound similar quantities of 125I-EPO. These data suggest that the erythrocytosis in these three patients was not mediated by a demonstrable alteration in EPO-binding characteristics.

**Paternity Tests**

The apparent autosomal dominant inheritance in families 2 and 3, and the lack of polycythemia in the parents or other relatives in family 1, raised the possibility of nonpaternity in the latter case. The results of paternity testing (Table 3) did not support this hypothesis, but suggest that the patient was a legitimate offspring of the parents. Based on the database collected for hMFl locus in a random population of white origin, this probability is 98.7%. The ABO and D blood groups were also found to be compatible with paternity, increasing the likelihood of this assumption.

**DISCUSSION**

In this study we have described three unrelated families with apparent familial and congenital forms of "primary" polycythemia. None of the subjects in any of the three families experienced any significant health impairment associated with their polycythemia. Some felt an improved sense of well-being after phlebotomy, while others did not. None of the polycythemic subjects in the three families had any evidence of secondary polycythemia, nor did they fulfill the diagnostic criteria of PV as outlined by the PV Study Group. All have been observed for at least 3 years (some for more than 9 years) and none have developed any of the long-term complications of PV such as myelofibrosis, spleno-megaly, anemia, thrombotic episodes, gout, pruritus, or leukemia. Furthermore, none have required any specific treatment for their polycythemia and their degree of erythrocytosis has remained remarkably stable, similar to the other two families that we reported previously in 1985.

To investigate the biologic mechanism underlying the polycythemia seen in these three families, we searched for two types of abnormalities: (1) those affecting the regulation of EPO production that would be manifested as an inappropriate increase in serum EPO levels, either at baseline or after phlebotomy; and (2) those affecting erythropoietic cell responsiveness to, or dependence on, EPO, which would be manifested as a change in the shape or position of the EPO dose-response curve of the affected progenitor class resulting in the formation of colonies containing mature erythroid cells in cultures to which no EPO has been added. In none of the three families studied here were increased EPO levels found. In family 1, as in the second of the two families reported previously, serum EPO levels were in the normal to low range. The other families all had subnormal EPO levels. In this respect, the patients with familial polycythemia resemble patients with PV whose polycythemia is also not associated with an increase in EPO production. However, in contrast to what is found in PV patients and also in normal historical controls, our subjects showed minimal or no increase in serum EPO levels in response to an approximate 20%
reduction in hemoglobin by phlebotomy. Previous reports demonstrate the inverse relationship between EPO and the hematocrit, which may become more significant as the degree of blood loss or anemia becomes more severe. The lack of response in our patients in the face of a 20% Hb drop almost mimics that seen in patients with EPO-producing tumors, although none of our subjects had tumors. These results clearly rule out the possibility that the polycythemia was the result of an abnormally sensitive oxygen sensing/EPO synthesis mechanism. In studying erythropoietic cell responsiveness and again in contrast to what is found in PV patients, none of the affected individuals described in this study showed evidence of a sub-population of erythroid progenitors with a clear-cut EPO-independent growth characteristic. In most patients with PV, about 10% to 50% of the CFU-E and BFU-E form colonies of mature erythroid cells containing detectable Hb in standard serum-containing cultures to which no exogenous EPO is added (ie, in which the EPO concentration is <0.001 U/mL), even in the presence of neutralizing antihuman EPO antibodies, and their numbers can be readily validated by full EPO dose-response curve analysis. Complete EPO dose-response data for CFU-E from one affected individual in family 1 were generated by two different laboratories at several times, the first occurring when the patient was age 2. Each time the data suggested that all of the CFU-E might have been slightly more sensitive to EPO resulting in a shift of the dose-response curve to the left. There were variations in the degree of the altered sensitivity at different timepoints from the two labs; however, at no point did the shift of the dose-response curve even approach that seen in PV. We observed similar growth characteristics in the other families with "primary" polycythemia. This is in direct contrast to the marked leftward shift of the dose-response curve observed in PV patients. Nevertheless, it is conceivable that clinically detectable polycythemia in these affected individuals might result from small but consistent increases in EPO-sensitivity not readily detected by the type of crude dose-response curve analyses to which the current technology of these progenitor studies is limited.

One explanation for increased sensitivity would be an alteration of the EPO-receptor. With the recent cloning of the human EPO-receptor, we were able to analyze this possibility by several sensitive methods. However, Southern blot analysis using two probes derived from the human genomic EPO-receptor showed no evidence for gene amplification, nor any gross chromosomal rearrangements, insertions, or deletions. Because our study was unable to identify an RFLP linked to the EPO-receptor, we cannot rule out the possibility of a minor change (point mutation) of the EPO-receptor from these individuals to search for EPO-receptor number and binding affinity in PV erythroid progenitors. Our results suggest that there are no receptor number or binding affinity changes in PV at least at the gross gene organization level. However, the possibility of more subtle mutations of the primary sequence leading to either functional alterations or transcriptional changes would not be shown by the Southern analysis. Additional complexities in PV pathogenesis are now appearing with the demonstration that PV BFU-E display a striking hypersensitivity to IL-3 and GM-CSF, in addition to EPO-hypersensitivity.

In summary, we believe that the polycythemic subjects in the three families investigated here, as well as possibly other families reported previously but not all fully characterized, exemplify a new type or class of polycythemia as outlined in Table 4. Other patients with familial and congenital polycythemia have either had elevated levels of EPO or have fit more closely a diagnosis of PV. In contrast, our patients had normal or low levels of EPO and, in two of these families, the cause(s) of polycythemia was inherited in an autosomal dominant fashion. The subject in family 1 in this report was an exception to this. In this case, the patient had normal parents and a normal brother, despite his apparently congenital polycythemia. This raises the possibility that this case involves either an autosomal recessive inheritance, an X-linked inheritance, or a new mutation (ie, spontaneous polycythemia). We have described previously two other unrelated white families residing in the southeast United States, in whom a phenotypically similar polycythemia appeared to be inherited in an autosomal dominant fashion. The documentation of a similar rare disorder prompted us to look for a common genetic lesion concentrated in one geographic area. However, to date, we have been unable to obtain any evidence
Types specified type. Other references were extensive gene linkage studies of these families may offer complementary approaches to explaining these disorders in more precise pathophysiologic and eventually molecular terms. Further studies of the intracellular events mediating the effect of EPO binding to its receptor and more extensive gene linkage studies of these families may offer complementary approaches to explaining these disorders in more precise pathophysiologic and eventually molecular terms.

NOTE ADDED IN PROOF
Since the submission of this report there has been another family, from Northern Europe, recently reported in Blood, that has many similar characteristics to the families reported in this manuscript. Also similar to our patients, Juvonen et al further stress that these subjects were asymptomatic and may not need any treatment of their polycythemia whatsoever. In fact, the propositus had even won several Olympic gold medals in cross-country skiing.

ACKNOWLEDGMENT
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